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14. ABSTRACT

The ongoing study addresses the critical clinical problem of posttraumatic joint stiffness, a pathology that reduces the range of motion (ROM) of injured joints and contributes to the development of osteoarthritis. The fundamental hypothesis that drives the current study is that pathological fibrotic response of injured joint tissues may be limited by targeting the formation of collagen fibrils, a main component of the fibrotic mass. Key preliminary data indicate the following: (i) in comparison to the non-treated control, deposition of newly-formed collagen fibrils in posterior capsules from injured knees of rabbits treated with the anti-fibrotic antibody is reduced significantly, (ii) in comparison to the non-treated control, the correct collagen III/collagen I ratio in posterior capsules from injured knees of rabbits treated with anti-fibrotic antibody is maintained, (iii) in comparison to the non-treated control, the ROM of injured knees of rabbits treated with anti-fibrotic antibody is greater. Ongoing studies with additional groups of animals will determine the statistical significance of the differences observed in the measured parameters. Completion of these experiments will define the utility of the anti-collagen I antibody to block excessive fibrosis associated with joint injury.

15. SUBJECT TERMS
Post-traumatic joint stiffness, anti-fibrotic therapy, collagen, therapeutic antibody, fibrosis, knee joint, animal model, range of motion.

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INTRODUCTION:

This annual report presents a summary of research activities during the second year of funding of a study entitled: "Prevention of the Posttraumatic Fibrotic Response in Joints". No changes were made to the original plan presented in the initial statement of work. The current study was awarded through the Translational Research Partnership Award mechanism to test a new approach to reducing trauma-associated stiffness of joints. The presented summary is a result of collaborative work done by a team of basic researchers and a group of orthopaedic surgeons. The ongoing study addresses the critical clinical problem of posttraumatic joint stiffness, a pathology that reduces the range of motion (ROM) of injured joints and contributes to the development of osteoarthritis. This clinical problem is highly relevant not only to military personnel, but also to the civilian population, and thus its medical significance is high.

The fundamental hypothesis that drives the current study is that the pathological fibrotic response of injured joint tissues may be limited by targeting the formation of collagen fibrils, a main component of the fibrotic mass. To test this hypothesis, we employ an engineered monoclonal antibody (Ab) that specifically targets collagen I molecules. By binding to the collagen molecules, the antibody prevents their self-assembly to fibrils, thereby reducing the growth of fibrotic tissue. We expect that this approach will reduce the flexion contracture and will shorten the time of full recovery. The current study utilizes a clinically-relevant model of post-traumatic joint stiffness. Specifically, this model employs rabbits which undergo a complex surgical procedure to cause trauma to the knee joint. During the two months of knee immobilization maintained with the use of a steel pin, the rabbits are treated with the anti-fibrotic antibody administered continuously for eight weeks directly to the knee cavity via a subcutaneous pump. Following the second surgery to remove the pin, the rabbits are allowed to recover for two or sixteen weeks. After that time, the rabbits are sacrificed, and the collected knees are then analyzed histologically, biochemically, and mechanically. Control groups include rabbits treated with the PEGylated version of the anti-fibrotic antibody, rabbits receiving weekly intramuscular injections of an anti-inflammatory agent (Depo-Medrol), and a group receiving only a solvent (PBS).

During the second year of this project, we continued the surgical procedures and subsequent assays of critical groups including rabbits with 2-week (Ab-2w) and 16-week (Ab-16w) recovery periods. In addition to our main group of rabbits treated with the anti-fibrotic antibody, we employed rabbits treated with a PEGylated form of this antibody and groups treated with an anti-inflammatory agent. Overall, the results obtained to date indicate beneficial effects of the therapeutic Ab on reducing the flexion contracture of experimental rabbits. In particular, beneficial effects were evident in the Ab-2w group. In this group the flexion contracture has decreased significantly in comparison to control in which no antibody was applied. In the Ab-16w group the flexion contracture has further decreased; no statistically significant differences were observed between the Ab-treated and the control group. This result is consistent with the earlier studies that demonstrated a spontaneous reduction of the joint flexion contracture over the 16-week period [1]. Hildebrand *et al*. have demonstrated that after that time the flexion contracture reached plateau and did not significantly change over an additional 16 weeks [1]. Considering our results for the Ab-2w and the Ab-16w groups, we postulate that the beneficial effect of the Ab is associated with the reduction of the formation of collagen deposits that contribute to the flexion contracture.

Our results with the groups treated with the anti-inflammatory agent (Depo) indicate a significant reduction of the flexion contracture. At the same time, however, application of this agent caused substantial side effects, including muscle loss, illustrated by a significantly greater loss of weight during treatment in comparison to the control.

Employing a PEGylated version of the antibody (P-Ab), we made a quite unexpected observation. The P-Ab version of the anti-collagen antibody was initially prepared by us as a control to serve as a "blocked" variant of the Ab. Our initial *in vitro* tests presented in the previous report clearly indicated the reduced binding of the PEGylated antibody to its collagen target (Fig. 1). Subsequent *in vivo* tests, however, demonstrated that the P-

Ab retains its blocking potential. Specifically, our results suggest that, despite its reduced binding to the targeted epitope, this antibody also significantly reduces the flexion contracture in the treated rabbits (Fig. 6). We postulate that PEGylation of the anti-fibrotic antibody may increase its stability, bioavailability, and prolong its residence time following delivery to the injury sites. Numerous studies on PEGylated therapeutic antibodies carried out by independent groups support this notion [2]. In addition to assays of control and fibrotic joint capsules collected to date, we plan to operate additional rabbits for the control group receiving only PBS and for the group receiving the PEGylated antibody. These additional tests will help to further investigate the utility of the P-Ab variant.

KEYWORDS:
Posttraumatic joint stiffness, anti-fibrotic therapy, collagen, therapeutic antibody, fibrosis, knee joint, animal model, range of motion.

OVERALL PROJECT SUMMARY:

Key participants:

This document offers a comprehensive report of research activities carried out by the involved PIs:

Andrzej Fertala, Ph.D., Initiating PI -- Dr. Fertala is an expert in the area of the structure and function of extracellular fibrous proteins with a special focus on collagenous proteins in health and disease. He provides critical input in the areas relevant to the effects of the proposed approach on the structure and functions of collagenous matrices formed in the presence of the tested molecules. Dr. Fertala oversees and coordinates the entire project and closely interacts with the other PIs and key members of the team.

Joseph Abboud, M.D. Partnering PI -- Dr. Abboud practices as a shoulder and elbow surgeon with a clinical emphasis on fracture care and trauma. His scientific interest includes posttraumatic contractures around the elbow. Dr. Abboud has conducted extensive basic science research with an emphasis on the biomechanical aspects of tendon healing and scar formation in the extremities using various animal injury models. Dr. J. Abboud performs animal surgeries. As a PI, due to his expertise in the field of orthopaedic mechanics, he is also critical for interpreting biomechanical tests of the studied joints. Dr. Abboud also participates in the intellectual process of conceptual design and critical analysis of the results of the performed studies.

Pedro Beredjiklian, M.D., Partnering PI -- Dr. Beredjiklian has a strong track record of basic research in examining the molecular basis of scar tissue and contracture formation after injury and surgery. Dr. Beredjiklian, a practicing orthopaedic surgeon, has a unique perspective on the proposed study. His role is complementary to that of Dr. Abboud, as the number of animals proposed here requires the involvement of two surgeons. In his PI role, Dr. Beredjiklian is responsible for the interpretation of clinically-relevant biological, histological, and morphological results of the analyzed joints.

The clinical observations of both surgeons are discussed with other participating surgeons and with the basic research investigators. In addition to the purely technical aspects of the project, broader intellectual involvement of the PIs is key for formulating new concepts and directions for the planned study. Such reciprocal communication is critical for moving the set scientific goals forward and for executing the planned study most productively, thereby ensuring a rapid transition of the proposed approaches from bench to bed. The flow of information is achieved via frequent communication among participating members.

Research activities:

In the second year of funding, no changes were introduced to the original plan of this study. All activities associated with our research activities paralleled those described in the initial SOW.

Our research activities followed the path defined in the specific aims and major tasks:

Specific Aim 1: "To block the fibrotic process after joint injury in a rabbit-based model"

Major Task 1: Production and purification of therapeutic antibodies.

The main elements of the **Major Task 1** were presented in detail in the first Annual Report. As indicated in the original SOW, the methods and procedures developed in the previous cycle of our research for antibody production will continue until the end of the 30th month of this project.

Blocking the activity of the anti-collagen I antibody- As described in the previous Annual Report, we prepared a version of antibody in which random lysine residues present in the Ab were bound via the ε-amino groups of the side chains to a 12-mer chain of polyethylene glycol (PEG; Pierce, Thermo Scientific). The N-hydroxysuccinimide (NHS)-based chemistry has been employed to achieve this binding. Subsequently, the modified anti-collagen I antibody was tested for its reactivity with collagen I via Western blot and biosensor-based assays, as described [3, 4]. Results of these earlier studies clearly show reduced binding (Fig. 1). Of interest however, is that the affinity of the PEGylated version remained very high (Fig. 3). We interpret these observations as follows: binding of PEGylated antibody molecules is reduced due to the steric hindrance imposed by the presence of PEG residues linked to lysine residues; the binding affinity of molecules that retain the ability to bind remains high.

Our subsequent *in vivo* assays (please see below) indicate that the PEGylated antibody retains the anti-fibrotic activity (Fig. 4&6). Thus, we propose that the reduced binding of the PEGylated variant is most likely compensated by its greater stability and retention in the knee cavity. This postulation is well supported by a number of independent studies showing that PEGylation of therapeutic antibodies may reduce their binding to specific targets, but the presence of PEG chains reduces proteolysis and enhances the retention time in target tissues (for review see [2]).

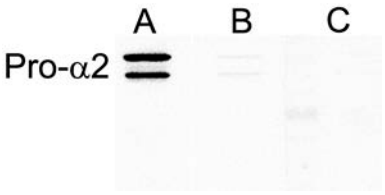

Figure 1. An original assay (presented in the first Annual Report) of the effectiveness of PEGylating the anti-collagen I antibody. A, Detection of the procollagen I-target (Pro-α2) with the non-PEGylated anti-collagen I antibody. B, A weak procollagen I-specific signal indicates steric hindrance imposed by PEG on the anti-collagen I antibody. C, Control Western blot done with the use of native human IgG with no specificity against collagen I.

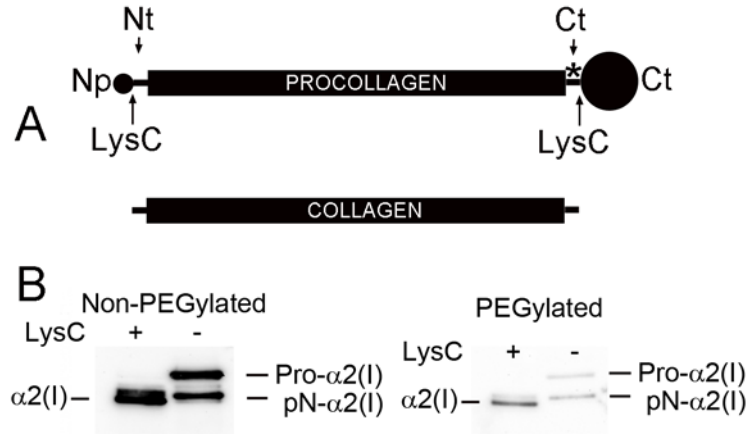

Figure 2. Western blot assay of the binding of the non-PEGylated and the PEGylated variants of the anti-collagen I antibody.

A, A schematic of key procollagen I domains: Np, Cp; procollagen N-terminal and C-terminal globular propeptides. Nt, Ct; procollagen N-terminal and C-terminal telopeptides. Note, that LysC cleavage of procollagen preserves the telopeptides. Asterisk indicates the antibody-binding site. A pN collagen variant represents a procollagen form in which the C-terminal

propeptides have been processed by enzymatic digestion. This form is frequently present in procollagen preparations. B, Western blot assays of the non-PEGylated and PEGylated antibody variants. Both variants recognize procollagen and collagen forms. Evidently, the binding of the PEGylated form is reduced due to mechanisms described above.

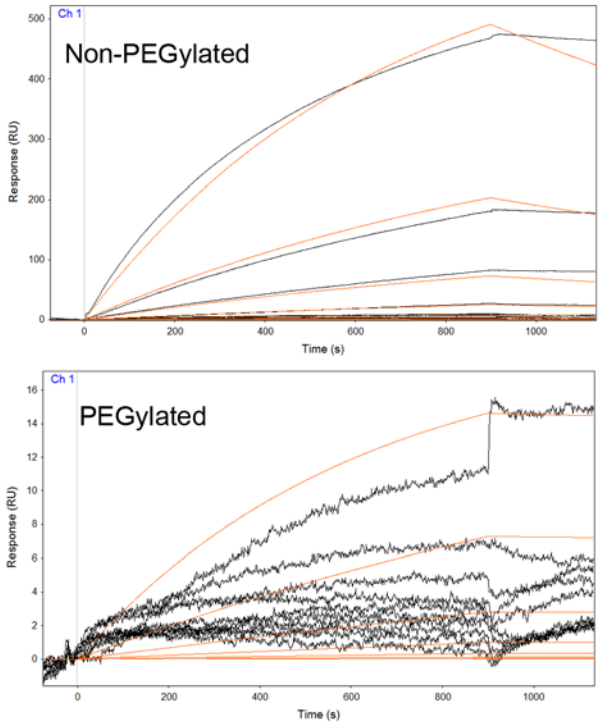

Figure 3 Biosensor assays of the binding of analyzed antibodies to the procollagen I target. Reduced response seen in the lower panel is a result of the steric hindrance imposed by the presence of PEG. The association and dissociation rates, however, are comparable to the non-PEGylated version.

Major Task 2: Testing procedures for generating a rabbit-based model of joint stiffness.

Currently, all tests and procedures associated with surgeries of rabbits to induce posttraumatic joint stiffness are well-developed and optimized. The detailed steps we employed to create all the key elements of the rabbit model of joint contracture are described in detail in our first Annual Report. These elements include rabbit surgeries, harvesting of legs, mechanical assays of the flexion contracture, and histological and biomechanical assays of the posterior knee capsules. A comprehensive description of the developed methodology and novel findings associated with pathomechanisms of posttraumatic joint contracture are described by us in a published article (please see attached copy) [5]. In accordance with the original SOW, we continue to utilize this model in our ongoing and future experiments.

Specific Aim 2: "To analyze long-term effects of the antibody-based inhibitor of fibrosis at the biochemical, cellular, and biomechanical levels"

Major Task 3: Creating an animal model for joint-contracture.

We have created all critical groups of rabbits. All surgical procedures and subsequent collection of tissues have been developed and are currently used on a regular basis.

Major Task 4: Evaluating the efficacy of inhibitory chIgG to reduce the consequences of traumatic joint injury.

During the second year of study, we successfully employed all assays needed to evaluate the utility of the inhibitory antibody to reduce the flexion contracture of injured knee joints. The employed techniques include mechanical assays of knee joints, microscopic observation of fibrotic tissue, and detailed analyses of intracellular processes that control fibrotic response due to joint injury.

The following paragraphs briefly described the results we have obtained to date. Note that upon completion of the ongoing experiments results will be updated with the incoming data.

Assays of collagen fibrils deposited in the posterior knee capsules (PC) – Since we target collagen fibril formation as a method to limit posttraumatic contracture due to the formation of fibrotic deposits, we analyze the relative content of various subpopulations of collagen fibrils present in the analyzed PCs. We employed a collagen-specific staining technique which combined with polarized light microscopy allows us to determine

the contribution of specific subpopulations of collagen fibrils [5]. In brief, the red/orange-colored fibrils represent thick, mature collagen fibrils and the green-colored fibrils indicate the presence of thin collagen fibrils. Yellow-colored structures represent intermediate-diameter fibrils. Studies demonstrated that in fibrotic tissue (F) with the active formation of new collagen fibrils, the relative content (fibrotic/uninjured control; F/C) of green-colored structures increases. Figure 4 indicates that in control injured knees (PBS group) the relative content of thin fibrils increases. In the Ab-2w group this increase was significantly smaller. In the Ab-16w group a similar trend was observed, but the differences between the corresponding PBS and Ab groups was not statistically significant. Both Ab and P-Ab variants caused similar responses. A relatively high value for the green-colored fibrils was observed in the 2-week recovery Depo group. At present we cannot interpret this result because to date we have only processed 2 rabbits in the Depo-treated group with a 2-week recovery period (Depo-2w). Data obtained to date indicate that the anti-collagen I antibody delivered directly to the site of knee injury reduces the amount of new collagen fibrils formed in response to trauma.

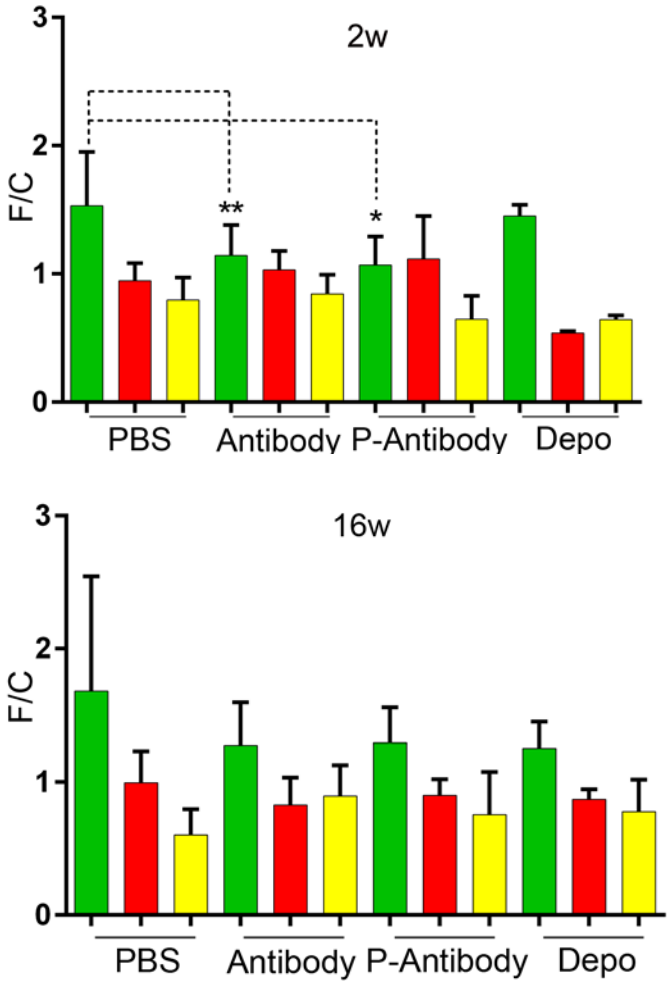

Figure 4. Microscopic assays of PCs from control and injured knees: Upper panel; 2 weeks, and lower panel; 16 weeks after surgery to remove a k-wire. Birefringence of picrosirius red-stained sections of control (E) and injured (F) PCs. G, Graphic representation of results of measurements of the F/C ratios of percent areas occupied by defined birefringence colors seen in fibrotic (F) and control (C) PCs.

Mechanical tests of the extension contracture – Following euthanasia, the legs of the rabbits were processed to remove the bulk of skin and muscle tissue [1, 5]. Then, the tibia and the femur were transected about 6 cm from the knee joint. Subsequently, the ends of the bones were potted in polycarbonate cylinders with the use of polymethyl methacrylate and acrylic copolymer (PMMA, Flexbar Machine Corp., Islandia, NY) (Fig. 5).

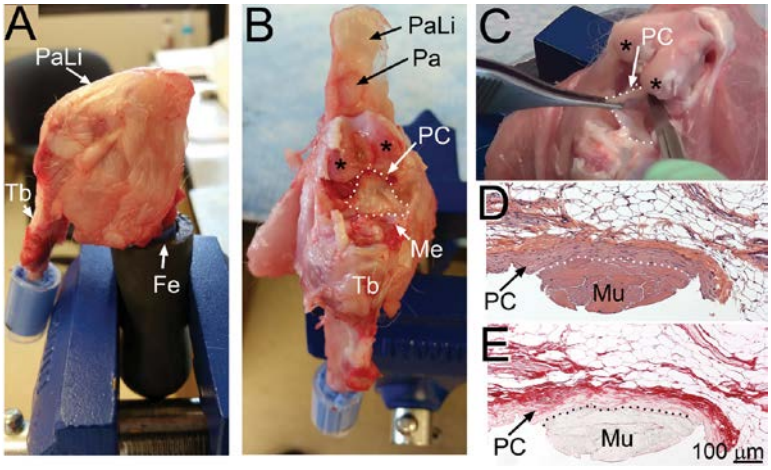

Figure 5. Dissecting the PC from a rabbit's knee. A, A medial aspect of the intact right knee. B, Anterior femoropatellar compartment of the analyzed knee. In panel B the anterior portion of periarticular tissues has been dissected to expose the PC, delineated by the dotted line. C, Dissecting the exposed PC, delineated by the dotted line; patellar ligament has been removed. D, E, Histological staining of the consecutive cross-sections of the dissected PC; in these panels the underlying muscle tissue has been purposely preserved to indicate the position of the PC. D, H&E; staining of the cross-section of the PC. E, Collagen-specific picrosirius staining of the PC observed in non-polarized light; in this panel the collagen-rich PC is clearly indicated by red staining. In D and E the net-like layer of fatty subintima of the synovial membrane is clearly apparent. The dotted lines in D and E mark the border between the PC and underlying muscle tissue. Symbols: PaLi; patellar ligament, Tb; tibia, Fe; femur, Pa; patella, Me; meniscus, Mu; muscle tissue. Asterisks indicate the femoral condyles.

Such processing ensured the secure placement of bones in the grips of a custom-made flexion-measuring device (Test Resources, Inc., Shakopee, MN); the device was designed with key technical parameters described by Hildebrand *et al.* [1, 6]. Apart from the gripping mechanism, this instrument included a torsional actuator/motor, a torque cell, and an angular displacement transducer. After securing the analyzed limb in the grips, the femur and tibia were positioned at a right angle, and then the instrument was set to 0° [1, 6]. Subsequently, applying the rate of loading set to 40°/min, an extension torque was applied to 0.2 Nm, and the joint extension was recorded [1, 6]. Values near 90° indicated maximum extension, while angles larger than 90° represented hyperextension. Flexion contracture was expressed as the difference between the angles of the control limb and the injured limb recorded at 0.2 Nm [1, 6]. Thus, a larger difference between the maximum extension angles of control and operated limbs indicates a more severe joint contracture.

The following groups of rabbits were analyzed: (i) rabbits treated with non-PEGylated anti-collagen I antibody for 8 weeks followed by a 2-week or 16-week recovery period (Ab-2w and Ab-16w), (ii) rabbits treated with PEGylated anti-collagen I antibody for 8 weeks followed by a 2-week or 16-week recovery period (P-Ab-2w and P-Ab-16w), (iii) rabbits treated with Depo for 8 weeks followed by a 2-week or 16-week recovery period (Depo-2w and Depo-16w), and (iv) rabbits receiving only PBS for 8 weeks followed by a 2-week or 16-week recovery period (PBS-2w and PBS-16w).

The mechanical tests indicate that treatment of the injured joints with both forms of the antibody was associated with reduced flexion contracture when compared to PBS control. While in the Ab-2w group the mean reduction of the flexion contracture was 14.69°, in the corresponding P-Ab-2w group, reduction was 32.05°. In the Depo-2w group the reduction was 57.09° (Fig. 6). As indicated in Fig. 6, in the 16-week recovery group further reduction of the flexion contracture occurred in all analyzed groups. In this group, no statistical

differences were observed between control and antibody-treated groups. A statistically significant difference between the flexion contractures of control and the Depo-16w group was observed (Fig. 6).

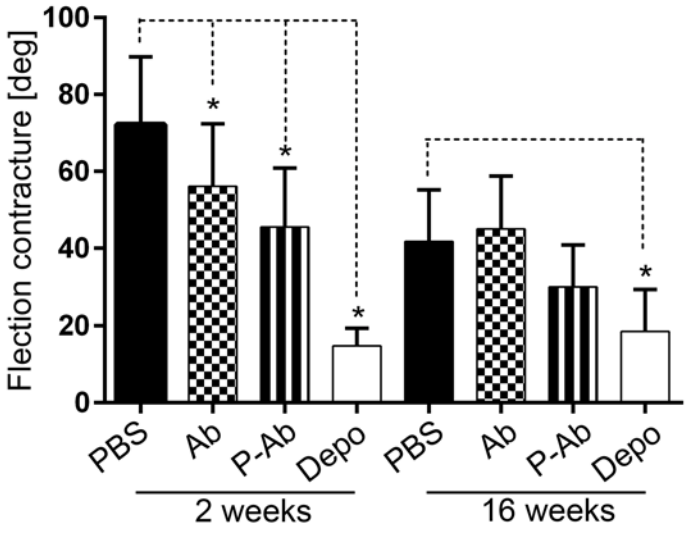

Figure 6. A graphic representation of the measurements of the flexion contracture of the injured knees. Note that following injury the analyzed knees were immobilized for 8 weeks. During this time the tested substances were delivered directly to the joint cavities via pumps. The flexion contracture was calculated as the difference between the flexion contracture of the non-injured leg and the flexion contracture of the injured leg. Note that a larger difference between the maximum extension angles of control and operated limbs indicates a more severe joint contracture.

Although the greatest reduction in the flexion contracture occurred in the rabbits treated weekly for 8 weeks with Depo, the treated rabbits experienced numerous side effects illustrated by the significantly greater loss of mass during the 8-week treatment in comparison to the PBS control (Fig. 7).

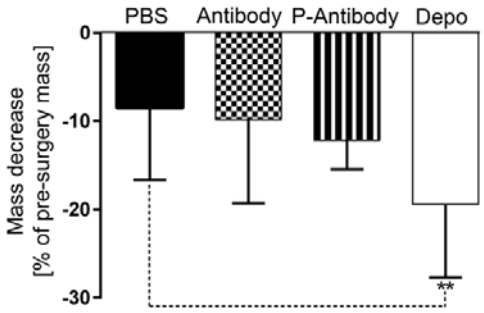

Figure 7. Changes in the body weight of rabbits during the initial 8 weeks of experimental study. During this time the rabbits were treated with tested and control substances.

Overall, our data to date indicate that treatment of the injured knees with the anti-collagen antibody reduces the joint contracture in the 2-week recovery group. Together with results indicating smaller deposition of new collagen fibrils in the studied PCs, we propose that the observed reduction of the flexion contracture was a result of antibody-mediated inhibition of collagen fibril formation. After 16 weeks of recovery, the flexion contracture is further reduced, but the differences between the PBS-16w group and the Ab-16w or P-Ab-16w are not statistically significant. After 16 weeks of recovery, the reduction of the flexion contracture plateaus, reaching similar values in all analyzed groups. The reduction of the flexion contracture in the knees of non-treated rabbits as a function of recovery time has been also observed in earlier studies. Specifically, studies done by Hildebrand *et al*. have demonstrated that the reduction of the flexion contracture plateaued 16 weeks after the second surgery to remove a pin, and it did not change by the end of the 32nd week [1].

Major Task 5: Task 4. Data analysis and statistical evaluation of results.

All data from the mechanical measurements, from the biochemical assays of collagen content, and from the quantitative histological assays are evaluated with the use of statistical tests.

KEY RESEARCH ACCOMPLISHMENTS:

- We defined critical mechanisms that control excessive production and deposition of fibrotic tissue in posttraumatic knee. These mechanisms include upregulation of heat shock protein 47 (HSP47), subunits of prolyl 4-hydroxylase, and lysyl oxidase (LOX).
- Contrary to the mainstream concept that increased formation of collagen cross-links is one of the key factors contributing to the development of fibrosis, our research indicates that in the posterior capsule of injured knee joint, the relative content of cross-links does not change. We also reported that in injured knees the organization of collagen fibrils is significantly altered [5]. This observation indicates that changes in the alignment of newly-formed collagen fibrils rather than their excessive cross-linking may be a key contributor to development of the posttraumatic joint contracture.
- Our data to date indicate beneficial effects of the tested antibody on reducing posttraumatic contracture in the rabbit model of knee injury.

CONCLUSION:

We have demonstrated that the employed animal model offers a biologically-relevant environment to study posttraumatic joint stiffness. The results of our study to date suggest that applying the anti-collagen I antibody to the injured knee reduces the amount of newly deposited collagen fibrils formed in response to the knee injury. Moreover, mechanical assays of the knee motion provide initial evidence that the flexion contracture in antibody-treated knees decreases as a result of such treatment.

PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS:

Our work has been presented at the Orthopaedic Research Society conference (March 2015) (see Appendix):

"Testing the Utility of Engineered Anti-Collagen I Antibody to Limit the Formation of Collagen-Rich Fibrotic Deposits in a Rabbit Model of Posttraumatic Joint Stiffness". Steplewski, Andrzej[1], Fertala, Jolanta[1], Barlow, Jonathan[1,2], Beredjiklian, Pedro[1,2], Abboud, Joseph[1,2], Wang, Mark[1,2], Namdari, Surena[1,2], Arnold, William[1,2], Kostas, James[1], Cheryl Hou[1], and Fertala, Andrzej[1]

[1]Department of Orthopaedic Surgery, Sidney Kimmel Medical College, Thomas Jefferson University, Philadelphia, PA, United States. [2]Rothman Institute of Orthopaedics, Thomas Jefferson University Hospital, Philadelphia, PA, United States.

Studies describing the fundamental processes associated with posttraumatic joint stiffness have been recently published by our group:

Steplewski A, Fertala J, Beredjiklian PK, Abboud JA, Wang ML, Namdari S, Barlow J, Rivlin M, Arnold WV, Kostas J, Hou C, Fertala A: Auxiliary proteins that facilitate formation of collagen-rich deposits in the posterior knee capsule in a rabbit-based joint contracture model, J Orthop Res 2015

INVENTIONS, PATENTS AND LICENSES:

None.

REPORTABLE OUTCOMES:

Research tool: An animal model to test the constant delivery of a therapeutic agent to the injured joint.
Effective antibody: Preliminary data indicate the beneficial influence of the Ab and the P-Ab variants on reducing joint contracture.

OTHER ACHIEVEMENTS:

This study provided a valuable training opportunity for a group of orthopaedic surgeons who are interested in translational approaches aiming at the development of a novel treatment. The following surgeons were attracted to and are actively involved in this study: Drs. Wang, Barlow, Namdari, Arnold, and more recently, Dr. Rivlin. Encouraged by the results of this study, approaches to reduce neural scarring are also prepared.

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APPENDICES:

An abstract submitted for the ORS conference.

Testing the Utility of Engineered Anti-Collagen I Antibody to Limit the Formation of Collagen-Rich Fibrotic Deposits in a Rabbit Model of Posttraumatic Joint Stiffness.

Steplewski, Andrzej[1], Fertala, Jolanta[1], Barlow, Jonathan[1,2], Beredjiklian, Pedro[1,2], Abboud, Joseph[1,2], Wang, Mark[1,2], Namdari, Surena[1,2], Arnold, William[1,2], Kostas, James[1], Cheryl Hou[1], and Fertala, Andrzej[1]

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A significant element of the pathomechanism associated with posttraumatic joint stiffness is fibrosis of joint tissues. Development of fibrosis is a complex process initiated in response to joint injury. In brief, the early process of inflammation attracts circulatory and resident cells, which secrete various growth factors. Consequently, these growth factors stimulate the migration of cells into the injury site and promote their proliferation. As a result, these cells actively produce various elements of the extracellular matrix needed to repair damaged joint tissues. In the fibrotic process, however, the synthesis and assembly of newly formed connective tissue is not balanced with the process of its degradation, thereby leading to the formation of pathological deposits of extracellular material. The main element of this fibrotic material is fibrils formed by the assembly of individual collagen I molecules. The excessive amount of collagen fibrils present in the fibrotic capsule contributes to the reduction of the range of motion (ROM) of the affected joint.

Because current approaches to reduce joint stiffness are not fully successful, our group formulated a hypothesis that collagen fibril formation, a key extracellular step in development of fibrotic deposits, represents an attractive target to reduce posttraumatic joint stiffness. The premise for this hypothesis is rooted in the fact that collagen fibril formation is a process driven by the self-assembly of individual collagen molecules and in the indication that blocking this process would reduce the mass of pathological fibrotic deposits, thereby reducing overall joint stiffness.

To test this hypothesis, we engineered an antibody that specifically recognizes and binds to a key domain of collagen I monomers, thereby preventing their assembly into fibrils. Our preliminary results done *in vitro* and in organotypic models of fibrosis indicated the potential utility of this antibody to limit collagen fibril formation [4, 7]. Here we present the novel preliminary results of our study, carried out in a rabbit-based model of posttraumatic joint injury. In brief, rabbits' knees were injured by employing a standardized technique, as described (Fig. 1) [6]. In each processed rabbit, the contralateral knee served as the uninjured control. Employing a k-wire, the injured leg was immobilized in a flexed position for eight weeks. During this time, the therapeutic antibody was continuously administered to the injury site via a pump installed subcutaneously (Fig. 1). Following eight weeks of immobilization, the k-wires were removed and the rabbits were allowed to move freely for two weeks. Subsequently, the rabbits were sacrificed and processed for mechanical, biochemical, and histological analyses (Fig. 1). First, employing a custom-made device (Fig. 1), the extension torque was applied to a maximum of 0.2 Nm, as described [6]. The extension angles were recorded to describe the degree of contracture of the analyzed knee joints. Next, the posterior knee capsules were dissected and processed for biochemical, histological, and immunohistological assays. The biochemical assays of collected tissues were performed to characterize the total collagen content, its composition, and degree of crosslinking. Moreover, tissue samples were stained with Sirius red and then collagen fibrils were studied with the use of a polarizing microscope to determine their quality and the extent of the fibrotic process in the analyzed samples. Specific immunohistology assays were conducted to characterize selected markers of the fibrotic process.

Mechanical analyses of the injured knees indicate severe contracture of the injured joints in the rabbit model employed here. The fibrotic morphology of the posterior knee capsules (Fig. 2B), and the presence of fibrotic markers such as connective tissue growth factor (CTGF, Fig. 2D), further demonstrated the development of the fibrotic tissue. No fibrotic changes were observed in uninjured contralateral knees (Fig. 2A, C). Polarized-light microscopic assays of collagen fibrils present in the healthy posterior capsules indicate that they comprise mature thick fibrils of a uniform morphology with a red/orange appearance. In contrast, the fibrotic capsules include numerous immature thin fibrils whose appearance is predominantly green (Fig. 2E).

The first-round analyses of the above parameters in a group of rabbits whose injured knees were exposed to the anti-collagen antibody demonstrated the reduction of fibrosis and the consequences of this reduction. Specifically, our initial mechanical tests indicate increased extension of the antibody-treated knees in comparison to the control. Specifically, our current data indicate that the average extension angle of the injured, non-treated knees was 24.3% of the non-injured contralateral control, while the corresponding value for the injured, antibody-treated knees increased to 37.3% ($p<0.05$). Our preliminary results on the quality and quantity of collagen fibrils present in the posterior capsules indicate that, in the injured rabbits not treated with the anti-collagen antibody, the relative content of the new fibrils formed as a result of the fibrotic processes was high (53.7%). In contrast, in the corresponding group treated with the anti-fibrotic antibody, the relative content of new collagen fibrils was markedly lower (25.8%). The content of thin, novel collagen fibrils in non-injured posterior capsules was 20.6% of the total collagen fibril content (Fig. 2E).

The biochemical assays do not indicate any significant changes in the total collagen content per unit of the dry mass of analyzed posterior capsules, thereby indicating that both non-fibrotic and fibrotic capsules have high collagen content of about 90% of their dry mass. Ongoing studies on the content of collagen fibril-stabilizing cross-links and the contribution of specific collagen types will provide further information on the effects of the anti-collagen antibody on the development of joint fibrosis.

In summary, our preliminary data indicate that the anti-collagen antibody that targets a key collagen I domain reduces the amount of newly-formed collagen fibrils in an injured joint capsule, thereby improving the ROM of an antibody-treated knee.

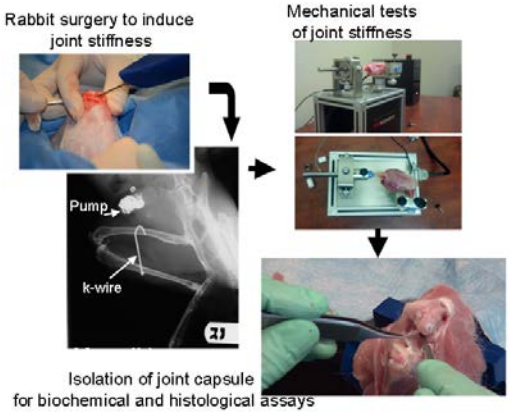

Figure 1. Illustration of critical steps of the described study.

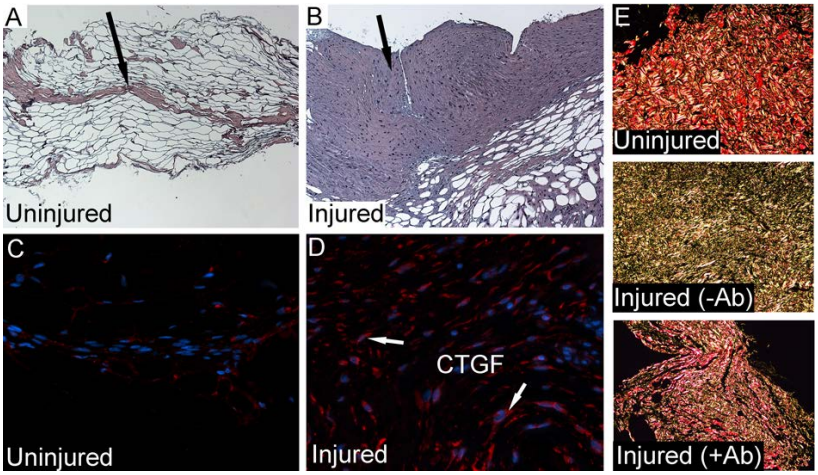

Figure 2. General morphology of the posterior capsule from an uninjured (A) and an injured knee (B); arrows indicate the structure of collagen-rich tissue. Immunostaining assays of CTGF indicate a background-level signal in a healthy posterior capsule (C) and markedly elevated presence of CTGF (arrows) in the fibrotic counterpart (D). E, A polarized-light image of the Sirius red-stained fibrils from an uninjured knee and from injured knees maintained in the absence (-Ab) or the presence (+Ab) of the anti-fibrotic antibody. Note that in the presence of the anti-collagen antibody, the amount of newly-formed fibrils (green) is markedly reduced.

Significance:

Posttraumatic joint stiffness represents a major medical problem. Because our study aims at developing new approaches to limit this problem, its significance is high.

Acknowledgements:
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A research paper published in the *Journal of Orthopaedic Research*:

Auxiliary Proteins That Facilitate Formation of Collagen-Rich Deposits in the Posterior Knee Capsule in a Rabbit-Based Joint Contracture Model

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ABSTRACT: Post-traumatic joint contracture is a debilitating consequence of trauma or surgical procedures. It is associated with fibrosis that develops regardless of the nature of initial trauma and results from complex biological processes associated with inflammation and cell activation. These processes accelerate production of structural elements of the extracellular matrix, particularly collagen fibrils. Although the increased production of collagenous proteins has been demonstrated in tissues of contracted joints, researchers have not yet determined the complex protein machinery needed for the biosynthesis of collagen molecules and for their assembly into fibrils. Consequently, the purpose of our study was to investigate key enzymes and protein chaperones needed to produce collagen-rich deposits. Using a rabbit model of joint contracture, our biochemical and histological assays indicated changes in the expression patterns of heat shock protein 47 and the α-subunit of prolyl 4-hydroxylase, key proteins in processing nascent collagen chains. Moreover, our study shows that the abnormal organization of collagen fibrils in the posterior capsules of injured knees, rather than excessive formation of fibril-stabilizing cross-links, may be a key reason for observed changes in the mechanical characteristics of injured joints. This result sheds new light on pathomechanisms of joint contraction, and identifies potentially attractive anti-fibrotic targets. © 2015 Orthopaedic Research Society. Published by Wiley Periodicals, Inc. J Orthop Res

Keywords: joint contracture; posterior capsule; collagen; collagen fibrils; arthrofibrosis; knee

Post-traumatic joint contracture is a common orthopaedic complication manifested by reduced range of motion.[1] Regardless of the nature of the initial injury, development of joint contracture includes: (i) inflammation, (ii) proliferation of activated cells, and (iii) enhanced production of extracellular matrix macromolecules that form collagen-rich fibrotic tissue that may be deposited in the intraarticular and periarticular areas.[2,3] These deposits are created by complex intracellular processes of biosynthesis of individual collagen molecules and their extracellular self-assembly.[4]

Upregulation of collagen production in the capsules of injured joints has been associated with joint contracture.[5–8] In spite of the critical role that collagen plays in fibrosis, the accompanying changes in key auxiliary proteins needed for posttranslational processing of collagen chains in fibrosis are poorly understood.

Each collagen molecule is composed of three collagen α-chains that associate in the endoplasmic reticulum (ER) into a triple-helical structure.[9] In the fibril-forming collagens, each chain consists of approximately 300 uninterrupted repeats of –Gly–X–Y– triplets. These collagens are produced as procollagens in which the triple-helical domains are flanked by globular N-terminal and C-terminal propeptides (Fig. 1). Relatively short telopeptides separate the propeptides and the triple-helical domain. Post-translational modification of selected proline and lysine residues of nascent collagen α-chains is a key step in procollagen biosynthesis. In particular, proline and lysine residues present in the –Y– positions of the –Gly–X–Y– triplets are hydroxylated by prolyl-4-hydroxylase (P4H) and lysyl hydroxylase (LH), respectively.[9] P4H is a tetramer consisting of two catalytic α units (P4Hα) and two non-catalytic β units (P4Hβ). P4Hβ also serves as protein disulfide isomerase (PDI). In addition to catalyzing the formation of disulfide bonds in procollagens and other proteins, PDI also functions as a protein chaperone that prevents premature aggregation of procollagen chains.[4,10] 3-hydroxyproline residues have also been found in the –X– and –Y– positions of the –Gly–X–Y– triplets, and the extent of 3-hydroxylation varies among different collagen types.[11] In procollagen I, however, only one proline residue of the α1(I) chains is 3-hydroxylated.[12]

Mature procollagen chains fold into a triple-helical structure in a zipper-like fashion.[13] By transiently binding to the procollagen chains, chaperone proteins stabilize them and prevent their nonspecific aggregation. Three key chaperone proteins are involved in procollagen folding: (i) heat-shock protein 47 (HSP47), (ii) heat-shock 70 kDa-related luminal binding protein (BiP), and (iii) P4Hβ/PDI.[14]

Upon secretion to the extracellular space, enzymatic cleavage of propeptides triggers collagen fibril formation.[15] A group of enzymes cleaves the N-terminal propeptides, including a disintegrin and metallopro-

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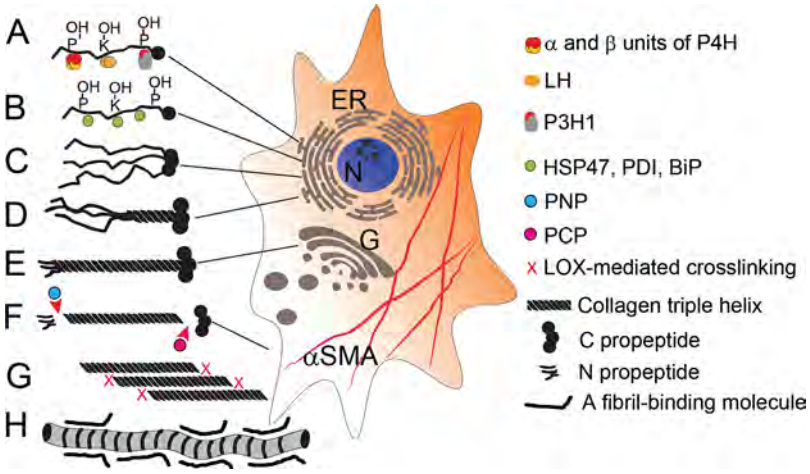

Figure 1. An illustration depicting intracellular and extracellular molecules and processes involved in the formation of collagen-rich deposits by fibrosis-associated cells. (A) Biosynthesis and post-translational modifications of individual procollagen α chains; during this process, selected proline and lysine residues are hydroxylated. Moreover, selected hydroxylysine residues may be further glycosylated. (B) The interaction of nascent procollagen chains with protein chaperones prevents nonspecific aggregation of individual chains. (C and D) Site-specific interaction of the C-terminal propeptides drives the selection of procollagen chains and initiates their folding into a triple-helical structure. (E) Intracellular translocation and secretion of procollagen molecules into the extracellular space. (F) Cleavage of the N and C propeptides by PNP and PCP, respectively. (G and H) Assembly of collagen molecules into fibrils in which formation of covalent cross-links stabilizes their structural integrity. ER, endoplasmic reticulum; G, Golgi apparatus; N, nucleus; αSMA, α smooth muscle actin.

tease with thrombospondin motifs (ADAMTS)-2, -3, and -14.[16] Enzymes belonging to the tolloid family of zinc metalloproteinases cleave the C-terminal propeptides of fibrillar procollagens, of which procollagen C proteinase (PCP) plays a pivotal role.[17] Procollagen propeptides may also be processed by meprins and mast cells chymase, enzymes whose activity increases during inflammation and fibrosis.[18,19]

Collagen fibrils form the main architecture of all elements of the musculoskeletal system. They self-assemble in a process driven by site-specific interactions among individual collagen molecules.[20] The covalent intramolecular and intermolecular cross-links stabilize the collagen fibrils. The hydroxylation of selected lysine residues and the catalytic activity of lysyl oxidase (LOX) facilitate the formation of collagen cross-links[4]

Collagen III co-assembles with collagen I to form heterotypic fibrils.[21] Studies indicate that collagen III regulates the diameter of the fibrils.[21] Researchers also showed that alterations of the collagen III:collagen I ratio may be responsible for changes in the morphology of collagen fibrils seen, for example, in Achilles tendinopathies and in the fibrotic skin of lipodermatosclerosis.[22,23]

In order to learn more about collagen formation after joint trauma, we analyzed the formation of collagen-rich deposits in the posterior knee capsules in a rabbit-based knee injury model. Since the role of the auxiliary protein machinery in the excessive production of collagen molecules is not well understood, we focused our efforts on characterizing contracture-associated profiles of its key elements.

MATERIALS AND METHODS

Joint Contracture Model

Animal studies were approved by our Institutional Animal Care and Use Committee (IACUC). We employed 10 female New Zealand White rabbits, 8- to 12-months old (Covance, Inc., Princeton, NJ) to study key aspects of the formation of collagen-rich deposits in a model of posttraumatic joint contracture. The clinical relevance of the model we selected is well established and its core design has been adapted by a number of independent research groups.[2,7,24–32] This model was employed as a part of an ongoing study that utilizes subcutaneous pumps to deliver compounds of interest to an injured rabbit. Here, we focused on the fibrotic processes in a group of untreated rabbits in which only phosphate buffered saline (PBS) was applied. Post-traumatic contracture was generated, as described, by simulating an intra-articular fracture (using drill holes) and a capsular injury (with a hyperextension injury) in the right knee and maintaining it in a flexed position using Kirschner (K)-wires for 8 weeks.[26,27] The contralateral uninjured limb served as control. Following the surgery, the rabbits were allowed unrestricted cage activity. After 8 weeks of immobilization, the K-wires were removed, and the rabbits were allowed an additional 2 weeks of unrestricted cage activity. After a 2-week recovery period the rabbits were euthanized.

Joint Angle Measurements

Following euthanasia, the legs of the rabbits were processed to remove the bulk of skin and muscle tissue.[6] Then, the tibia and the femur were transected about 6 cm from the knee joint. Subsequently, the ends of the bones were potted in polycarbonate cylinders with the use of polymethyl methacrylate and acrylic copolymer (PMMA, Flexbar Machine Corp., Islandia, NY) (Fig. 2). Such processing ensured the secure placement of bones in the grips of a custom-made flexion-measuring device (Test Resources, Inc., Shakopee, MN); the device was designed with key technical parameters described by Hildebrand et al.[6,27] Apart from the gripping mechanism, this instrument included a torsional actuator/motor, a torque cell, and an angular displacement transducer (Fig. 2). After securing the analyzed limb in the grips, the femur and tibia were positioned at a right angle, and then the instrument was set to 0°.[6,27] Subsequently, applying the rate of loading set to 40°/min, an extension torque was applied to 0.2 Nm, and the joint extension was recorded.[6,27] Values near 90° indicated maximum extension, while angles larger than 90° represented hyperextension. Flexion contracture was expressed as the difference between the angles of the control limb and the injured limb recorded at 0.2 Nm.[6,27] Thus, a larger difference between the maximum extension angles of control and operated limbs indicates a more severe joint contracture.

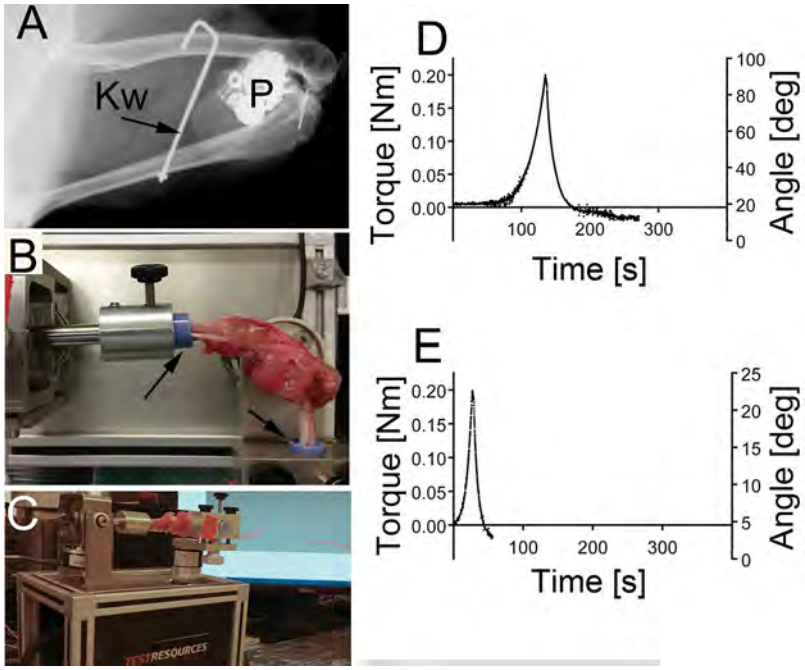

Figure 2. A rabbit-based model of joint contracture. (A) An X-ray image of the operated knee. A k-wire (Kw) and a pump (P) delivering PBS are depicted. (B and C) Views of a system employed to measure the flexion contractures of analyzed knees. In B, arrows indicate femur and tibia whose ends are potted into tubes with the use of a PMMA. (D and E) Representative data from measurements of the flexion contractures of the healthy (D) and the contracted (E) knees at 0.2 Nm torque. Ascending and descending parts of the depicted graphs represent extension and flexion behavior of the analyzed knees.

Tissue Collection

As the fibrosis of the posterior joint capsule (PC) is the main cause of contracture in the rabbit model employed here, following the flexion contracture measurements, the PCs were dissected from the limbs, as described (Fig. 3).[6,29,33]

Total Collagen Content

Tissue samples were frozen in liquid nitrogen and then pulverized in a stainless steel mortar. The samples were treated with a 3:1 mixture of chloroform and methanol to extract lipids and then lyophilized and weighed. Next, a hydroxyproline assay was used to determine the collagen content per unit of dry mass.[34]

Collagen III: Collagen I Ratio

First, the acid-soluble collagen fraction was extracted from collected tissues with the use of 0.5 M acetic acid. Subsequently, the pepsin-soluble fraction was obtained with the use of pepsin (Sigma–Aldrich, St Louis, MO) prepared in 0.5 M acetic acid at a final concentration of 1 mg/ml. Employing the Sircol Collagen Assay Kit (Biocolor Ltd., Carrickfergus, UK), the concentration of extracted collagen was determined, and then the samples were prepared for electrophoresis. To separate the monomeric forms of collagen I chains from collagen III monomers, we employed an interrupted electrophoresis method that takes advantage of the fact that, unlike the collagen I α-chains, the collagen III α-chains are bound together via reducible disulfide bonds.[35] In brief, heat-denatured collagen samples were initially run in a 6% polyacrylamide gel in non-reducing conditions. After 15 min, a 10-μl aliquot of 0.5 M DTT was added to each well and then electrophoresis continued. Recombinant collagen III served as a marker (Fibrogen, Inc., San Francisco, CA). Pixel intensities of protein bands corresponding to the α1(III) and the α2(I) chains were measured by densitometry (EZQuant Ltd., Tel-Aviv, Israel), and then collagen III:

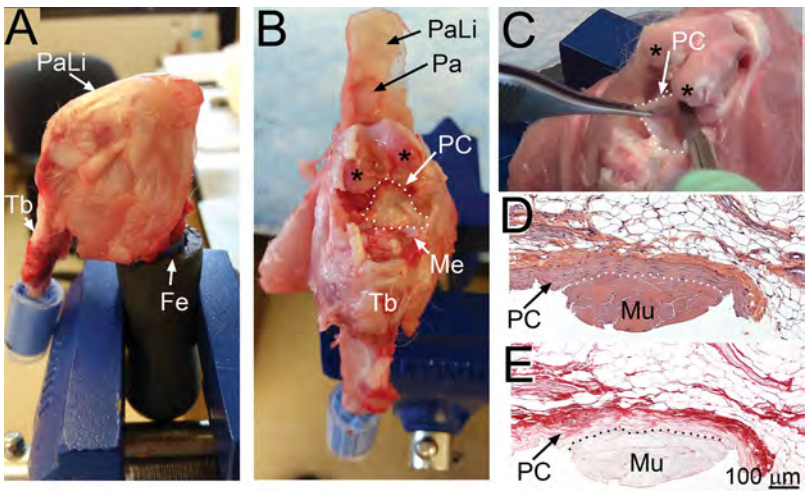

Figure 3. Dissecting the PC from a rabbit's knee. (A) A medial aspect of the intact right knee. (B) Anterior femoropatellar compartment of the analyzed knee. In panel B the anterior portion of periarticular tissues has been dissected to expose the PC, delineated by the dotted line. (C) Dissecting the exposed PC, delineated by the dotted line; patellar ligament has been removed. (D and E) Histological staining of the consecutive cross-sections of dissected PC; in these panels the underlying muscle tissue has been purposely preserved to indicate the position of the PC. (D) H&E; staining of the cross section of the PC. (E) Collagen-specific picrosirius staining of the PC observed in non-polarized light; in this panel the collagen-rich PC is clearly indicated by red staining. In D and E, the net-like layer of fatty subintima of the synovial membrane is clearly apparent. The dotted lines in D and E mark the border between the PC and underlying muscle tissue. PaLi; patellar ligament, Tb; tibia, Fe; femur, Pa; patella, Me; meniscus, Mu; muscle tissue. Asterisks indicate the femoral condyles.

collagen I ratios were calculated. The ratios of cross-linked α-chains forming the β and the γ oligomers to the monomeric α-chains of the collagen pool extracted from PCs were also analyzed with the use of densitometry.

Microscopy of Collagen Fibrils

Three-micrometer thick sections of paraffin-embedded PCs were stained with H&E and with collagen-specific picrosirius red (Polysciences, Inc., Warrington, PA). The latter staining technique combined with polarized-light microscopy makes it possible to describe the thickness, the organization, and to a certain degree, the collagen type-specific composition of the fibrils.[36] As the thickness of fibers increases, their birefringence color changes from green to yellow to orange to red, that is, from shorter to longer wavelengths.[36–38] Employing a polarizing microscope (Eclipse LV100POL, Nikon Inc., Melville, NY) and the NIS Elements software (Nikon Inc., Melville, NY), we determined the percentages of differently colored collagen fibrils, as described.[39] Applying the built-in settings of the NIS Elements software, three main groups of colors were defined: (i) green, (ii) yellow, and (iii) orange-red. The entire image was analyzed, and then the areas occupied by pixels corresponding to the defined colors were determined. Considering the sum of all pixels to be 100%, the percentage of each color group in the analyzed samples was calculated. A minimum of three histological sections for control and for injured PCs were analyzed per each rabbit. The organization of collagen fibrils, expressed as theoretical anisotropy score, was evaluated by employing the ImageJ software that included the FibrilTool plug-in.[40,41]

Immunohistology

The main focus of the quantitative immunohistological assays done with paraffin-embedded samples was to analyze the changes in key proteins associated with the biosynthesis of collagen deposits formed in analyzed PCs. Immunohistology was also employed to visualize selected markers of the fibrotic process. Table 1 lists all antibodies employed in our study and indicates the experimental conditions applied. In addition, all samples were exposed to 4′,6-diamidino-2-phenylindole (DAPI) to visualize the nuclei.

In all assays, the specimens from injured and healthy PCs were processed simultaneously to ensure identical conditions for immunostaining. A fluorescence microscope (Eclipse E600, Nikon, Inc.) equipped with a digital camera (DS-Qi1Mc, Nikon, Inc.) was employed. Adopting a strict protocol for the quantitation of the pixel intensity, values assured the accuracy of measurements of the immunostained specimens.[42] For quantitative assays, a minimum of three sections of the PCs isolated from the injured and control knees were employed to measure each parameter. The mean pixel intensity values derived from measurements of immunostained cells observed in separate viewing areas of analyzed samples were calculated and presented individually as scatter plots. These values were compared to control set at 100%.

Statistical Analyses

For each parameter analyzed in a PC isolated from an injured knee ($n = 10$), a corresponding parameter for the contralateral PC ($n = 10$) served as control. For histology-based assays, data from multiple sections of a capsule were analyzed. The statistical significance of differences between the control group mean and the experimental group mean has been evaluated with the use of the Student's *t*-test (GraphPad Prism v. 5.03, GraphPad Software, Inc., La Jolla, CA).

RESULTS

Joint Contracture Model

Consistent with earlier reports, all surgically operated knees developed flexion contracture.[6,27] The mean value for the flexion contracture, defined as the difference between the flexion angles at 0.2 Nm measured for control (92.3°, ± 10.4 SD) and for operated limbs (22.4°, ± 7.7 SD), was 69.9° (± 7.5 SD) (Fig. 2D and E).

Observations of transverse sections of uninjured PCs indicate that this structure is built by the layer of a fibrous, compact connective tissue (Fig. 4A) and the adjoining net-like layer of fatty subintima of the synovial membrane (Fig. 4B). Compared to the control, the fibrous layers of the capsules isolated from the injured knees were noticeably thicker and their apparent cellularity has increased (Fig. 4A and C). In addition to the fibrous layer of the capsule, accumulation of atypical tissue deposits within subintima was also clearly apparent in the specimens from the injured knees (Fig. 4B and D).

Microscopic Assays of Collagen Fibrils

Picrosirius red staining (Fig. 4E, F, H, and I) revealed changes in the percentages of green, yellow, and orange-red sub-populations of fibrils. Specifically, in comparison to 22.1% (± 7.4 SD) of green-colored fibrils seen in the control, the percentage of corresponding fibrils increased to 32.5% (± 9.4 SD) in injured PCs. This increase was accompanied by a decrease in the percentages of the yellow-colored and the orange-red-colored fibrils (Fig. 4G). We also demonstrated changes in the organization of collagen fibrils. Specifically, observations of longitudinal sections of the PCs revealed a defined, fishbone-like pattern, a result of crimps in parallely-organized fibrils (Fig. 4H). In contrast, such an arrangement was not readily apparent in the fibrotic capsules (Fig. 4I). Instead, the fibrils were distributed more randomly through the entire area of the analyzed PCs. The mean value for the theoretical anisotropy score calculated for the fibrils seen in the healthy capsules was 0.26 (± 0.07 SD) while the corresponding value for the injured capsules was 0.09 (± 0.04 SD).

Total Collagen Content

Assays of hydroxyproline content indicate a relatively high amount of collagen in the PCs isolated from the healthy and contracted knees. In the healthy knees, the total collagen contributed 0.92 (± 0.14 SD) mg per 1 mg of dry mass of the PC. Similarly, in the capsule from the contracted knee, collagenous proteins contributed 0.88 (± 0.15 SD) mg per 1 mg of dry mass. There was no statistically significant difference between these two values ($p = 0.4$).

Table 1. Primary and Secondary Antibodies Used to Detect Selected Targets

Target	Antigen Recovery: Buffer Incubation Conditions	Primary Antibody: Manufacturer Catalog Number Dilution; Incubation Conditions	Secondary Antibody: Manufacturer Catalog Number Dilution; Incubation Time; Temperature
CTGF	Citrate buffer 70°C; 2 h	Santa Cruz Biotechnology, Dallas, TX sc-14939 1:200; 2 h; RT	Thermo Fisher Scientific, Rockford, IL anti-Goat-Alexa Fluor© 594; A11058 1:1,000; 1 h; RT
P4Hα	Citrate buffer 70°C; 2 h	LifeSpan Biosciences, Inc., Seattle, WA LS-B3291 1:200, 1 h; RT	Molecular Probes anti-Goat-Alexa Fluor© 594; A11058 1:1,000; 1 h; RT
P4Hβ	Citrate buffer 70°C; 2 h	LifeSpan Biosciences, Inc., Seattle, WA LS-B3137 1:200; 1 h; RT	Thermo Fisher Scientific, Rockford, IL anti-Mouse-Alexa Fluor© 594; A11032 1:1,000; 1 h; RT
HSP47	Citrate buffer 70°C; 2 h	Santa Cruz Biotechnology, Dallas, TX sc-5293 1:200; 2 h; RT	Thermo Fisher Scientific, Rockford, IL anti-Mouse-Alexa Fluor© 594; A11032 1:1,000; 1 h; RT
BiP	Citrate buffer 70°C; 2 h	LifeSpan Biosciences, Inc., Seattle, WA LS-B4157 1:200; 1 h; RT	Thermo Fisher Scientific, Rockford, IL anti-Mouse-Alexa Fluor© 594; A11032 1:1,000; 1 h; RT
Mast cells chymase	No antigen retrieval step	Acris Antibodies, Inc., San Diego, CA SM1536P 1:2,000, 1 h; RT	Thermo Fisher Scientific, Rockford, IL anti-Mouse-Alexa Fluor© 594; A11032 1:1,000, 1 h; RT
LOX	Citrate buffer 70°C; 2 h	Santa Cruz Biotechnology, Dallas, TX sc-48723 1:500; 1 h; RT	Thermo Fisher Scientific, Rockford, IL anti-Goat-Alexa Fluor© 594; A11058 1:1,000; 1 h; RT
LH1	Citrate buffer 70°C; 2 h	Santa Cruz Biotechnology, Dallas, TX sc-271640 1:500; 1 h; RT	Thermo Fisher Scientific, Rockford, IL anti-Mouse-Alexa Fluor© 594, A11032 1:1,000; 1 h; RT
α-SMA	0.1% trypsin in 5 mM $CaCl_2$, 37°C; 30 min	Abcam, Cambridge, MA ab-7817 1:200; 2 h; RT	Thermo Fisher Scientific, Rockford, IL anti-Mouse-Alexa Fluor© 594; A11032 1:1,000; 1 h; RT
Laminin β2	0.1% trypsin in 5 mM $CaCl_2$, 37°C; 30 min	EMD Millipore, Billerica, MA 05-206 1:200; 2 h; RT	Thermo Fisher Scientific, Rockford, IL anti-Rat-Alexa Fluor© 488; A11006 1:1,000; 1 h; RT

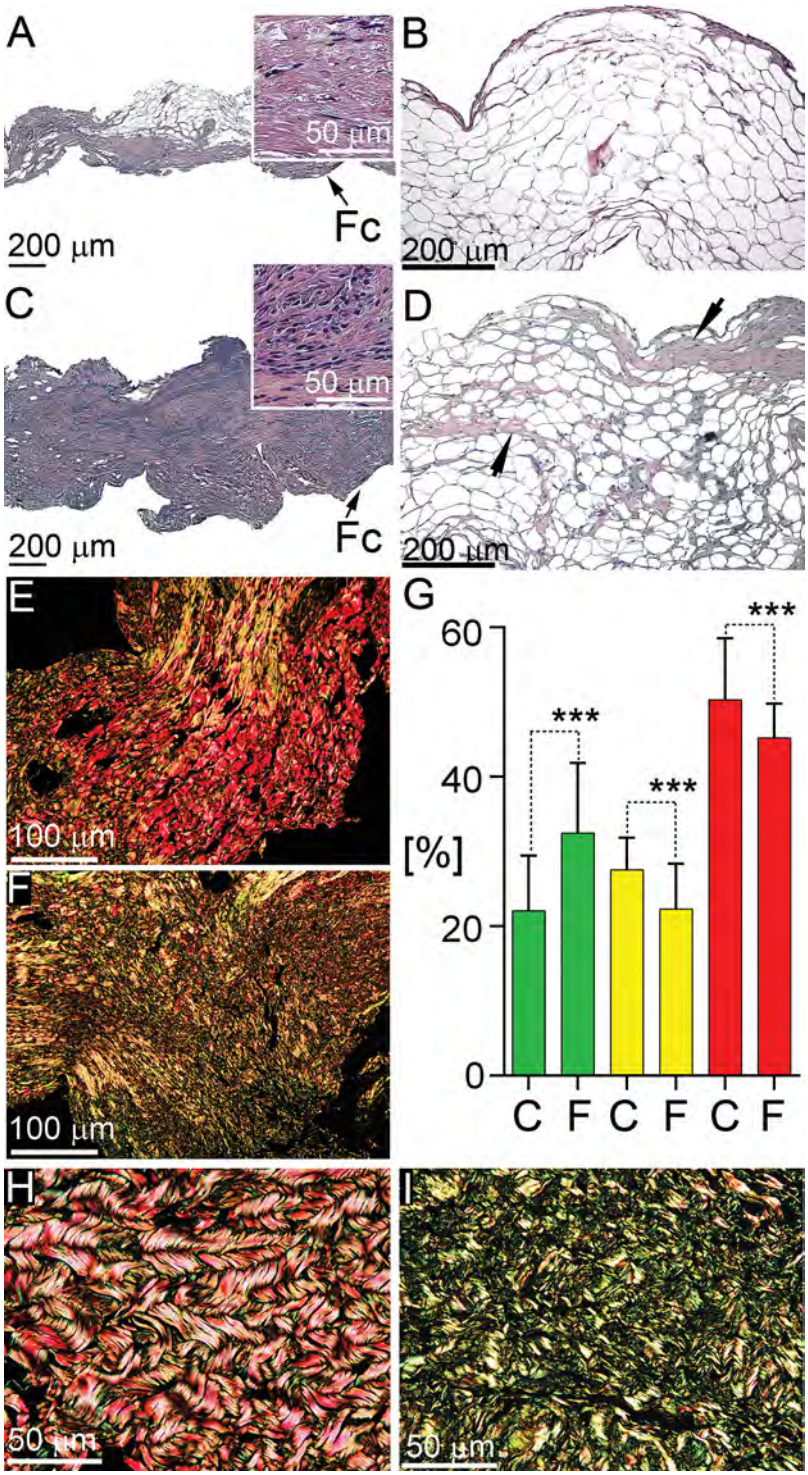

Figure 4. Microscopic assays of PCs from control and injured knees. (A and B) H&E staining of cross-sections of a fibrous capsule (Fc) (A) and subintima (B) of control PC. (C and D) H&E staining of cross-sections a fibrous capsule (C) and subintima (D) of injured PC. The inserts presented in A and C indicate the apparent difference in the overall cellularity of the capsules. In D, arrows indicate fibrotic deposits within subintima. (E and F) Birefringence of picrosirius-stained cross-sections of control (E) and injured (F) PCs. (G) Graphic representation of results of measurements of percent areas occupied by defined birefringence colors seen in control (C) and fibrotic (F) PCs ($^{***}p < 0.001$). (H and I) longitudinal sections of the control (H) and injured (I) PCs stained with picrosirius. In H, the fishbone-like pattern of the organization of collagen fibrils is clearly apparent. In contrast, collagen fibrils seen in I are randomly distributed.

Electrophoretic Assays of Collagen

Densitometry assays show that the mean collagen III:collagen I ratio for control knee capsules is 0.78 (± 0.18 SD), while the corresponding value for the injured capsules is 0.98 (± 0.21 SD), a statistically significant difference ($p = 0.01$) (Fig. 5B). In contrast, as indicated by a similar $\beta\gamma/\alpha-$chains ratios, there was no difference between the relative amounts of the cross-linked chains present in the pools of the pepsin-extracted collagens from control and experimental groups ($p = 0.7$) (Fig. 5C). As the amount of acid-extracted collagen was low (not shown), this collagen fraction was not further analyzed. Moreover, as collagen chains from control and fibrotic PCs co-migrated in the electrophoretic field, no overmodification, that is, overhydroxylation and overglycosylation, of collagen chains from fibrotic tissues was present.

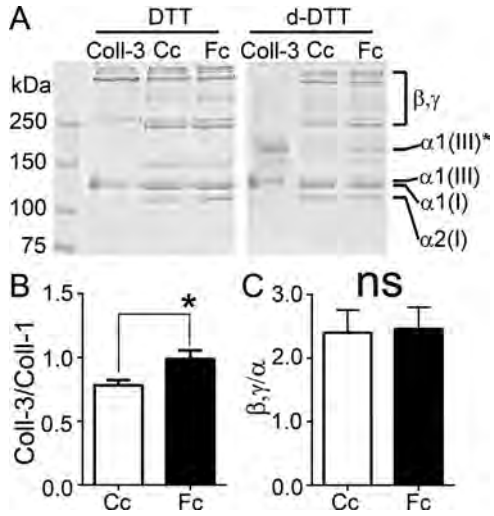

Figure 5. Electrophoretic assays of collagenous proteins extracted from control capsule (Cc) and fibrotic capsule (Fc). (A) Patterns of migration of collagen I and collagen III chains separated in standard reducing conditions (DTT) and in delayed-reduction conditions (d-DTT) of electrophoresis. (B and A) Graphic representation of the results of assays of collagen III: collagen I ratios ($^*p < 0.05$). (C and A) Graphic depiction of the results of measurements of βγ:α ratios; no statistically significant difference was observed. Coll-3; collagen III marker, α1(I), α2(I), α1(III); specific chains of collagen I and collagen III, respectively; α1(III)*; collagen III chains separated with the use of delayed-reduction conditions; β, γ; β and the γ oligomers formed consisting of cross-linked collagen α chains.

Selected Markers of Fibrosis

In addition to the changes seen in the collagen-rich extracellular matrix, the fibrotic process in the injured PCs was demonstrated by qualitative immunohistological assays (Fig. 6). First, the α smooth muscle actin (αSMA)-positive staining in fibroblastic cells seen in the fibrous (Fig. 6A) layer of fibrotic PCs was clearly apparent. These cells were readily distinguishable from the αSMA-positive and laminin β2-positive smooth muscle cells present in blood vessels (Fig. 6B). Control capsules were largely negative for the αSMA-specific staining (not shown). Second, we demonstrated that, unlike in the control, cells present in the injured capsules stain positively for connective tissue growth factor (CTGF) (Fig. 6C and D). Third, the chymase-specific staining demonstrated the presence of mast cells in the injured but not the control PCs (Fig. 6E and F). No immunostaining was detected in the negative controls in which the primary antibodies were omitted (not shown).

Proteins Associated With Collagen Production

Measurements of the pixel intensities of fluorescence signals associated with the analyzed proteins revealed their expression profiles in cells present in the injured PCs (Figs. 7–9). Specifically, a threefold increase was observed for the catalytic P4Hα subunit and for HSP47 (Figs. 7, 8, and 9). The increase in the P4Hβ/PDI subunit was 1.5-fold, while relative to the control, the amount of the LOX and BiP increased 1.8-fold and 1.2-fold, respectively (Figs. 7, 8, and 9). There was no statistically significant change in the relative amount of LH1 in the analyzed cells (Figs. 7 and 9). Background-levels of fluorescence signals in the negative controls validate the specificity of immunostaining in the positively-stained cells (not shown).

DISCUSSION

In this study, we focused on fibrotic changes taking place in the PC of rabbits' knees. Although intraarticular fibrotic adhesions may be formed as a result of trauma, Hildebrand et al. have demonstrated that fibrosis of the PC is the main cause of joint contracture in the rabbit model employed here.[6,29] Since the biosynthesis of extracellular collagen-rich deposits requires the involvement of auxiliary protein chaperones and collagen-modifying enzymes, the increase in collagen content seen in the fibrotic joint capsules cannot be explained simply by the increased production of collagen chains. Consequently, we studied critical elements of a biological system that allow efficient processing of collagen molecules produced in response to joint injury. The development of severe flexion contracture, increased cellularity, and the presence of fibrosis-specific αSMA-positive, CTGF-positive, and chymase-positive cells in the injured PCs validate the biological relevance of the employed model.[2,7,43,44] Our assays of the total collagen content indicate that the healthy and fibrotic PCs are collagen-rich tissues. Assays of the injured capsules demonstrated that at the end of a 2-week recovery period, there was a significant increase in the content of green-colored fibrils. This result suggests that the population of new fibrotic tissue formed in the injured capsules includes relatively thin fibrils. These collagen fibrils could represent a pool of fibrils undergoing lateral aggregation into large-diameter fibers similar to those seen in the control. These fibrils, however, could also be the end-product of the fibril formation process. Considering such a possibility, we propose that collagen III may act as the fibril diameter-limiting factor. This notion is supported by earlier observations that collagen III limits fibril growth through co-polymerization with collagen I.[21] Moreover, researchers studying the decrease in the diameter of fibrils seen in tendinopathies have suggested that an increased collagen III:collagen I ratio contributed to such a change.[23] A similar increase in the collagen III: collagen I ratio was also associated with a decrease in the diameter of collagen fibrils observed in the fibrotic skin of lipodermatosclerosis.[22]

To further elucidate mechanisms that enable efficient processing of the increased amount of collagen in fibrotic capsules, we analyzed collagen-modifying enzymes and protein chaperones. In the first category of molecules, the most prominent increase was noticed in catalytic P4Hα and non-catalytic P4Hβ/PDI subunits of P4H. Although the P4Hβ unit does not directly participate in the catalytic activity of P4H, its

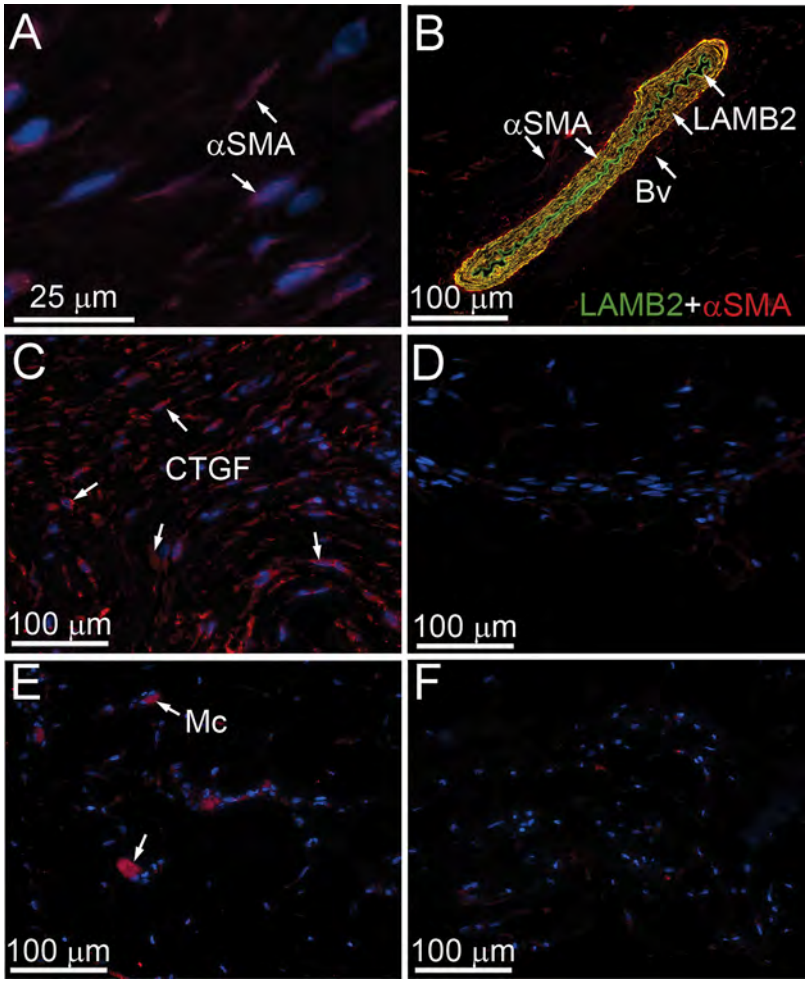

Figure 6. Immunostaining assays of selected markers of fibrosis in the cross-sections of analyzed PCs. (A)Visualization of αSMA (arrows) in cells present in the injured PC. (B) Depiction of a blood vessel (Bv) that stains positively for αSMA and β2 chain of laminin (LAMB2). This picture clearly distinguishes αSMA-positive fibroblastic cells from the αSMA-staining seen in Bv (arrows). (C and D) Staining of fibrotic (C) and control (D) PCs for CTGF. (E and F) Staining of fibrotic (E) and control (F) PCs for mast cells (Mc) chymase. Arrows indicate the chymase-positive cells clearly apparent in panel E.

presence is needed to anchor this enzyme in the ER and to maintain the catalytically active structure of its P4Hα subunit. As the presence of nascent collagen chains is a prerequisite for the assembly of stable αα/ββ tetramers, we believe that increased production of collagen chains promotes the association of individual subunits into active P4H.[45] Such a system provides an effective regulatory mechanism for P4H, thereby ensuring proper post-translational modifications of collagen chains and their folding into thermostable molecules.

In contrast to the increased expression of P4H in fibrotic PCs, the expression of LH1, an enzyme that catalyzes the hydroxylation of certain lysine residues in the triple-helical domain of collagens, did not change in comparison to the control. This result is consistent with a study of the keloid-derived fibroblasts in which researchers demonstrated that expression of the LH1 variant at the mRNA level was similar to that in control cells.[46] Moreover, no statistically significant LH1 expression changes were seen in fibroblasts derived from Dupuytren's lesions.[46] Normal expression of LH1 was also consistent with the lack of apparent overmodification of collagen chains, a process dependent on the overhydroxylation of lysine residues. These results are also consistent with studies reported by Akeson et al. who reported no changes in the hydroxylysine/lysine ratio in periarticular tissues surrounding contracted rabbits' joints.[24] Based on our studies, however, we cannot exclude the possibility of an increased production of LH2, a telopeptide lysyl hydroxylase variant whose expression changes in certain fibrotic tissues.[46]

In this study, a steady amount of LH1 was accompanied by a 1.8-fold increase in LOX production. LOX plays a critical role in maintaining the integrity of collagen-rich tissues as the enzyme catalyzing the formation of allysine and hydroxyallysine. Allysine and hydroxyallysine formation are key steps in the formation of the covalent cross-links between fibril-incorporated collagen molecules.[47] The expression of LOX, however, varies in different fibrotic tissues. For instance, mRNA-based studies of the expression of LOX in cells derived from keloids, hypertrophic scars, hepatic stellate cells, and fibroblasts derived from Dupuytren's lesions have demonstrated that a significant, twofold increase of LOX expression was seen only in the last group of cells.[46] Based on the electrophoretic

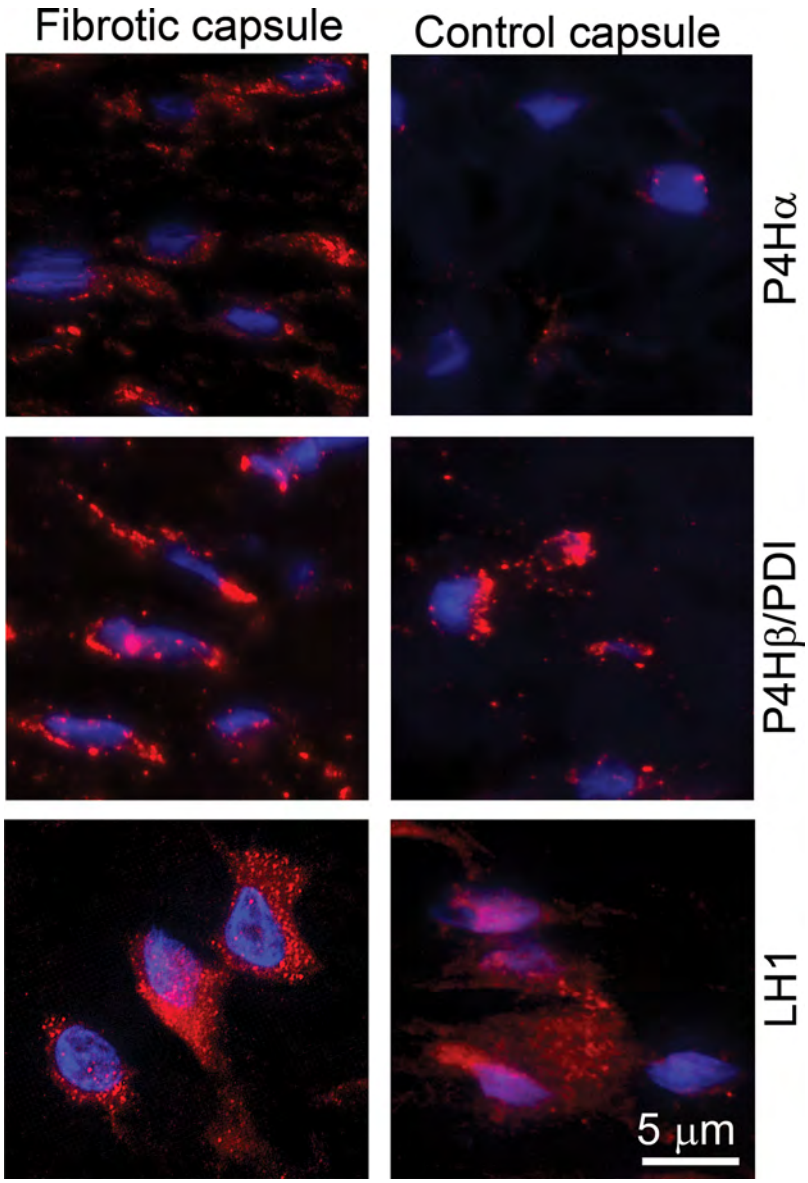

Figure 7. Immunostaining of cross-sections of fibrotic and control PCs for P4Hα, P4Hβ/PDI, and LH1.

measurements of the cross-linked versus non-cross-linked collagen chains, we determined that the relative amounts of the cross-linked fractions in the injured and healthy capsules were similar. As in our assays, we employed the pepsin-soluble fraction of the collagen pool, we cannot exclude the possibility of changes in the quality and the quantity of cross-links present in the non-soluble fraction. Akeson et al. have demonstrated an increase in the quantity of cross-links in collagen isolated from periarticular tissues isolated from immobilized rabbits' knees. Unlike our studies in which we specifically isolated the PCs of affected joints, Akeson et al. isolated periarticular tissues that included fascia, ligaments, and capsule. Employing these tissues, the authors demonstrated that the major cross-links that increased are hydroxylysinonorleucine, dihydroxylysinonorleucine, and histidinohydroxymerodesmosine types.[48]

Our microscopic assays revealed alterations of fibril organization seen in longitudinal sections of the injured joint capsules. Specifically, we demonstrated that the dominant fishbone-like pattern of organization of the fibrils seen in control capsules changes to a random distribution of fibrils synthesized during fibrosis. Such a random orientation of the fibrils could result from prolonged inactivity of the injured joints and ongoing tissue remodeling that includes degeneration of collagen fibrils. The importance of the mechanical forces for the tissue-specific alignment of fibrils is a well-recognized phenomenon. In 1951, MacConaill concluded that "as iron filings are to a magnetic field so are collagen fibrils to a tension field."[49] Since then, the role of mechanical forces in fibril organization was demonstrated in connective tissues such as meniscus, tendon, ligament, and others.[50,51] Researchers have demonstrated that the force-fibril orientation relation-

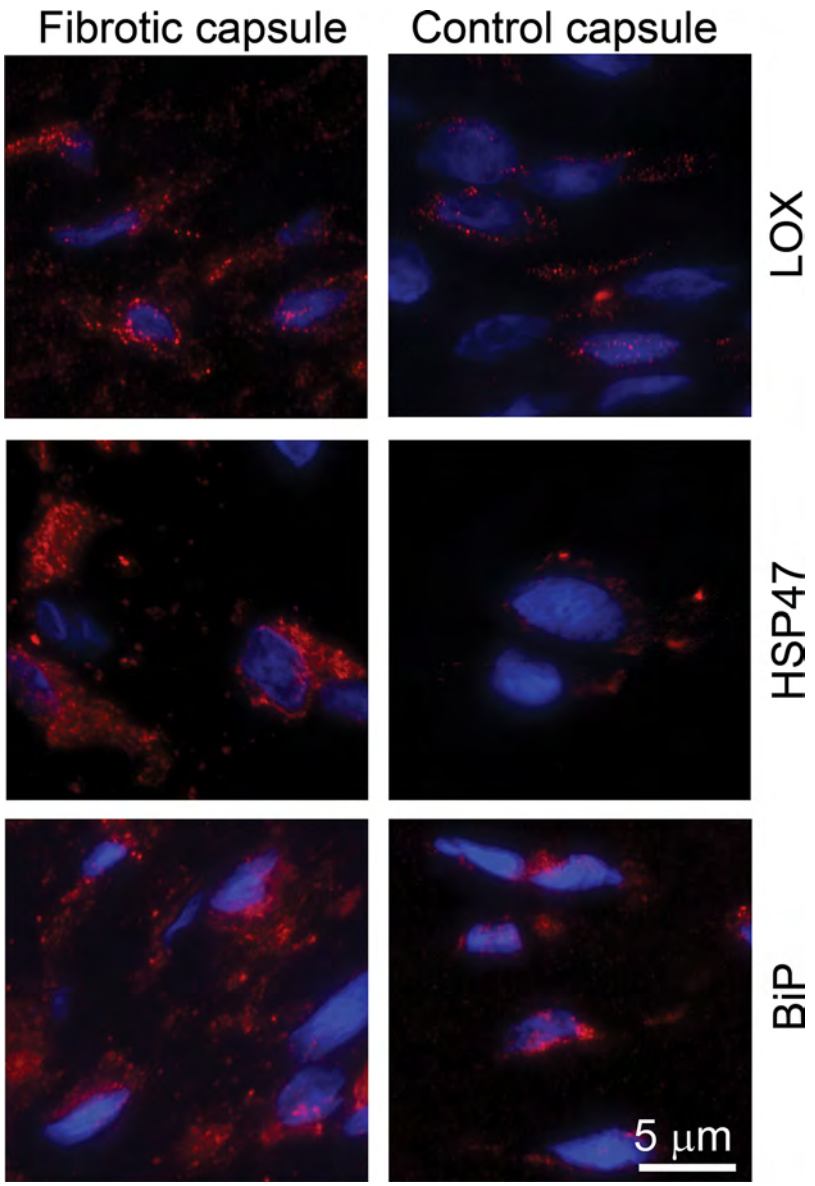

Figure 8. Immunostaining of cross-sections of fibrotic and control PCs for LOX, HSP47, and BiP.

ship is a key parameter that defines micro-structural properties in all of those tissues. Based on these considerations, we believe that abnormal fibril orientation rather than excessive cross-link formation contributes to altered mechanical properties of injured capsules. In support of this postulation is a recent study of muscle fibrosis reported by Chapman et al. In this study, researchers have demonstrated that the stiffness of skeletal muscles of experimental mice could not be explained by increased collagen cross-linking. Indeed, the authors suggested changes in collagen fibril orientation as a possible decisive factor in developing the muscle stiffness.[52] Similar suggestions on the potential role of aberrations of the micro-architecture of collagen fibrils in joint contracture were made in early studies carried out with the use of a rabbit-based model.[25]

To further elucidate the mechanisms that facilitate the excessive deposition of collagen-rich matrix in PCs, we analyzed protein chaperones that play a critical role in the intracellular processes of the biosynthesis of collagen molecules. Among the chaperones analyzed (PDI, BiP, and HSP47), the expression of HSP47 increased most significantly.

Our demonstration of the increased production of HSP47, a key collagen-specific chaperone, indicates that this protein plays a critical role in maintaining the proper folding of collagen molecules in the fibrotic PC. Numerous reports of increased HSP47 production in fibrotic tissues that include skin, cornea, liver, and tendon adhesions further highlight the critical role of this chaperone in maintaining the fibrotic process.[53] Thus, the increased production of HSP47 and, to a lesser extent, other chaperone proteins seen in our study may represent a common defense mechanism protecting fibrotic cells from ER stress-associated apoptosis. Indeed, experimental deletion of HSP47 in hepatic stellate cells causes excessive intracellular

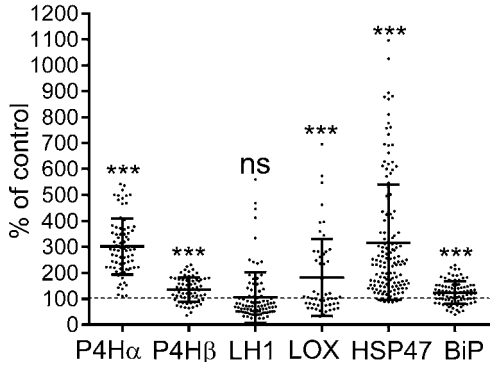

Figure 9. Graphic representation of relative intracellular content of P4Hα, P4Hβ/PDI, LH1, LOX, HSP47, and BiP. The mean values (± SD) seen in scatter plots represent changes in indicated proteins present in cells seen in the injured PCs. Each point in the plot represents the mean value calculated for the group of cells seen in the particular viewing area of analyzed specimens. The dotted line, set at 100%, represents the corresponding values calculated for control cells observed in non-injured PCs (***$p < 0.001$, ns; not significant).

accumulation of misfolded collagen molecules which leads to ER stress and cell death.[54]

The processing of the N-terminal and the C-terminal propeptides is prerequisite for collagen fibril formation. Studies in induced liver fibrosis demonstrated that the activity of PCP increases due to upregulation of the expression of procollagen C–proteinase enhancer protein (PCPE).[55] Similarly, Hultström et al. demonstrated a significant increase in the expression of PNP in a rat model of renal fibrosis and clearly associated this increase with excessive accumulation of collagen-rich deposits.[56] These data suggest that upregulation of PNP and PCP is needed in fibrosis to ensure effective conversion of procollagen into fibril-forming collagen molecules. Although detailed assays of procollagen processing are beyond the scope of this study, it is worth noting the potential role of the mast cells chymase in the removal of procollagen propeptides. As chymase processes both procollagen propeptides in a way that enables collagen molecules to assemble into proper fibrils, we cannot exclude the possibility that this enzyme may contribute to excessive production of fibrotic deposits seen in PCs of contracted joints.[19]

Due to inherent limitations of our animal-based model of joint contracture, such as anatomical differences and specific injury type, results of our study may not fully represent human pathology associated with joint contracture. By following the fundamental steps in the path of fibrillar collagens, however, our study sheds new light on the fundamental mechanisms that control excessive production of fibrotic deposits. Specifically, our data indicate that increased production of the P4H subunits and upregulation of the biosynthesis of HSP47 are critical for the intracellular processing of excessively produced collagen molecules. Moreover, the data emphasize a potential role of mast cells in extracellular processing of procollagens, shedding new light on a possible role of these cells in propagating fibrosis. In addition, detailed assays of newly formed collagen fibrils in injured joint capsules indicate that their altered organization, rather than atypical crosslinks, is a potentially critical contributor to the observed changes in the flexion contracture. Taken together, our study extends knowledge on the pathomechanisms of post-traumatic joint contracture, thereby contributing to developing new concepts for potential therapy approaches.

AUTHORS' CONTRIBUTIONS

A. Steplewski and J. Fertala: Substantial contribution to the acquisition, analysis, and interpretation of data. Contributed to drafting the manuscript. A. Fertala, P. Beredjiklian, J. Abboud, and J. Barlow: Substantial contribution to research design and execution of key experiments. M. Wang, S. Namdari, M. Rivlin, and W. Arnold: Substantial contribution to the acquisition and interpretation of data form an animal model. J. Kostas and C. Hou: Substantial contribution to the acquisition and interpretation of data. All authors have read and approved the final submitted manuscript

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